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Effect of diethylnitrosamine on the anisotropy of the liposomal membranes

Shashiprabha Dasgupta, Dipak Dasgupta*, Anindita Chakraborty* and Papiya Nandy†

Department of Physics, Jadavpur University, Calcutta-700 032, India
* Biophysics Division, Saha Institute of Nuclear Physics,
37 Belgachia Road, Calcutta-700 037, India
*Bioscience Division, IUC, DAE, Calcutta-700 091, India
E-mail : anandy@vsnl.com

Abstract : Presence of proper fluidity is necessary for the highly specific functions of the biological membranes and this can be manipulated by external agents like different chemicals, drugs *etc* Diethylnitrosamine (DENA) is one of the most potent members of the carcinogenic nitrosamines, which induces tumours in kidney, liver *etc*.. In order to reach its target DNA, DENA has to pass through the cell membrane and in so doing it would affect membrane properties. However, till now there is no report on the effect of DENA on this aspect

Here, we report our study on the effect of DENA on the fluidity (measured in terms of anisotropy) of the liposomal membrane of dipalmitoyl phosphatidylcholine (DPPC) and that containing DPPC and cholesterol, using a fluorescent polarization probe as the reporter molecule The result indicates that DENA interacts with these liposomal membranes only when cholesterol is incorporated in them and there is a change in the vesicle size associated with the interaction

Keywords Membrane anisotropy, diethylnitrosamine

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1. Introduction

Membrane fluidity plays a very important role in determining several functional properties of membrane, like entry of food and drug in cell, excretion of unwanted products, cell fusion, invasion by bacteria, virus *etc*. [1]. This fluidity can be manipulated by the presence of external agents of chemical (like drugs *etc*.), biological (bacteria, virus or other diseases) or physical (temperature, pH, *etc*.) origins. Carcinogens affect the membrane structure and function to a considerable extent. A major proportion of human cancers have been estimated to be of environmental origin such as those caused by smoking and occupational carcinogens [2]. N-nitrosodialkylamines are a group of powerful carcinogens that have been shown to cause a wide range of tumors in all animal species tested so far [3]. These compounds, being present in the work place, processed meats, cigarette smoke and beverages, pose a potential health hazard to humans [4]. In view of the ubiquitous occurrence of their precursors, N-nitrosodialkylamines may contribute substantially to the growing incidence of cancers in humans.

† Correspondence Author

Diethylnitrosamine (DENA) forms one of the most potent members of the carcinogenic nitrosamines. It has been found to induce tumors in kidney, liver, esophagus and respiratory tract of various experimental animals [3]. Like other nitrosamines, DENA is assumed to require metabolic activation in the host organism to exert its carcinogenic effect. The tissue-specific enzymatic N-dealkylation of DENA to an electrophillic intermediate which is capable of ethylating DNA is thought to be responsible for the mutagenic properties of this chemical [5]. Replication of such damaged DNA may lead to somatic mutation and cancer development. In order to reach its target DNA, the carcinogenic compound DENA has to pass through the cell membrane and in doing so, it would affect the membrane properties, fluidity being one of them. But how this compound affects the membrane before penetration into the cell has not been studied earlier.

Using liposomal membrane of the lipid dipalmitoyl phosphatidylcholine (DPPC), a system that is extensively used as a model for the highly complicated natural membranes, we report here for the first time how DENA affects some of the physical properties like fluidity (measured in terms of anisotropy)

and chain melting temperature. We have used a fluorescent polarization probe 1,6 – diphenyl – 1,3,5-hexatriene (DPH) as the reporter molecule. Our study shows that DENA affects the membrane properties only when cholesterol is present in the membrane and associated with it there is a change in the vesicle size.

2. Methods

2.1. Preparation of DENA incorporated liposome

The liposome of DPPC and DPPC containing cholesterol in 5 mM Tris-HCl., pH 7.5, buffer were prepared as follows. The lipids in chloroform were dried by rotary evaporation to form a thin film. This was suspended in buffer and sonicated using a bath sonicator (Imeco ultrasonicator UP 250F) to obtain a unilamellar suspension of vesicles. The sample was stored at 4°C for 16 hrs and once again sonicated for 30 mins before use [6]. The final concentration of the lipid was 0.1 mM. The molar ratio of lipid : cholesterol was 1 : 0.25.

2.2. Fluorescence anisotropy measurements

DPH (1,6-diphenyl-1,3,5-hexatriene) dissolved in N, N-dimethyl formamide was added to the sonicated suspension of liposome to a final concentration of 0.5 μM (lipid : DPH in 1 : 0.005 molar ratio) and incubated for 15 min at 40°C. To this was added DENA (lipid : DENA in 1 : 0.5 molar ratio) and incubated for 3 hrs at 25°C. The steady state fluorescence anisotropy of DPH in liposome is a function of the membrane viscosity and is given by the following equation [6]

$$r = (I_1 - I_2) / (I_1 + I_2),$$

where, I_1 and I_2 are the vertical and horizontal components of the 428 nM emission band of DPH in the liposome while the sample is excited by vertical component of light at 360 nM. In all experimental set-ups, the fluorescence intensities were calculated after properly eliminating the light scattering effect. A Perkin Elmer spectrofluorimeter was used to measure the steady state fluorescence anisotropy. All the experiments were done in thermostated cell maintained at a constant temperature. The values reported here are an average of five independent experiments with a standard deviation of 5 %.

2.3. Electron microscopic studies

An aliquot of the suspension of free DPPC liposome containing cholesterol and DENA treated DPPC containing cholesterol liposome were used for the preparation of the grids at 25°C. Negative staining method was used for viewing the pictures. From both gel filtration and electron microscopic studies, the average size of the DPPC liposomes were estimated to be 250 ± 40 Å. For this study, a Hitachi H-transmission electron microscope operating at 75 KV was used.

3. Results and discussion

In Figure 1, we have shown the effect of DENA on the anisotropy of DPH probed DPPC liposomal membrane in the presence and in the absence of cholesterol. The result shows that DENA does not affect the fluidity profile of DPPC liposome. However, when DENA is incorporated in the DPPC liposome in presence of cholesterol, the fluidity profile gets modified.

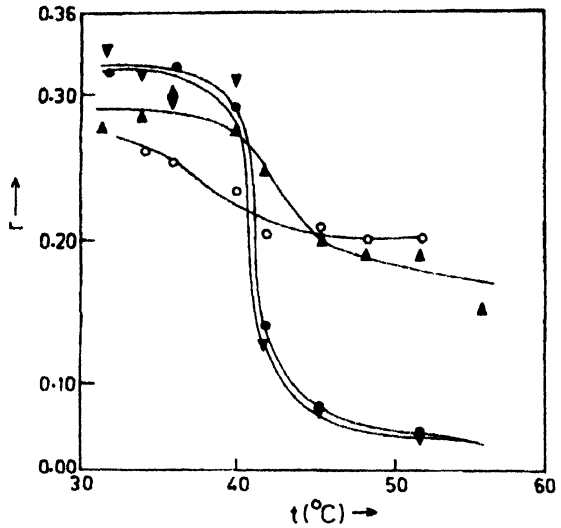

Figure 1. Fluorescence study of the effect of DENA on the anisotropy of liposomal membranes of (a) DPPC (10^{-4} M) ▼-▼, (b) DPPC DENA (in 1 0.5 molar ratio) ●-●, (c) DPPC cholesterol (in 1 0.25 molar ratio) ▲-▲, (d) DPPC cholesterol · DENA (in 1 0.25 · 0.5 molar ratio) o-o

All physical measurements characterize the role of cholesterol on lecithin liposome as one of producing "an intermediate fluid condition" of the membrane [7]. With increase in cholesterol level, there is an increase in the value of anisotropy at higher temperature and decrease in this value at low

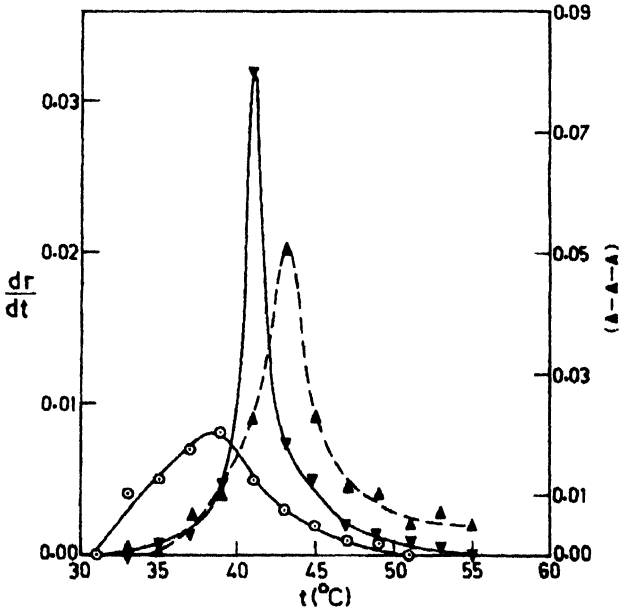

Figure 2. Derivative plot (dr/dt) for the curves presented in Figure 1. (a) DPPC (10^{-4} M) ▼-▼, (b) DPPC : cholesterol (in 1 : 0.25 molar ratio) ▲-▲, (c) DPPC : cholesterol : DENA (in 1 : 0.25 : 0.5 molar ratio) o-o, Data presented here are the mean of five separate experiments with standard deviation of 5%. The scale for the derivative plot of curve representing DPPC only (a) has been denoted on the right hand side of the graph.

temperature in the interior of the lecithin liposomes [8]. Our results show that DENA changes the fluidity profile of the cholesterol incorporated DPPC liposome. In presence of DENA, the membrane becomes more fluid in the lower temperature region and more rigid in the higher temperature region of the cholesterol incorporated membrane and the corresponding phase transition temperature shifts to a lower value from 42.5°C to 39°C as determined by derivative analysis [Figure 2]. This indicates that DENA probably gets localized in the cholesterol rich domain in the DPPC-cholesterol liposomes.

The electron microscopic study shows that DENA also has a fusogenic effect on the liposomes containing cholesterol [Figure 3]. This increase in size perhaps facilitates the entry of DENA into the cell. As liposomes are well accepted model for studying the much too complex biological membranes, this study is quite significant in determining the role of DENA on fluidity profile of the liposomal membrane. Our study shows that DENA affects the membrane structure besides altering the properties of DNA in the process of making a normal cell cancerous. Though this *in vitro* study has more physical significance than a physiological one, the results obtained here may be used to interpret the effects that are observed *in vivo*.

Figure 3. Electron microscopic study of the effect of DENA on the vesicle size of liposomal membranes of (a) DPPC . cholesterol (in 1 0 25 molar ratio), (b) DPPC cholesterol . DENA (in 1 0 25 0 5 molar ratio) The magnification was 50,000 × ,1 cm = 2000 Å

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